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13. ABSTRACT (Maximum 200 Words) The link between diet and breast cancer has been long postulated and recently, extensively investigated. Identifying a molecular mechanism linking diet and breast cancer, however, has remained elusive. Recently, we identified an isoform of the cellular receptor called the peroxisomal proliferator-activated receptor (PPAR) in several human breast cancer cell lines. Activation of PPAR can alternatively lead to tumor induction or differentiation into a more benign state, depending on the tissue and the isoform expressed. We and others have demonstrated that human breast cancer cell lines express PPAR α and that individual fatty acids are capable binding to and functioning as selective agonist or antagonist of PPAR. Furthermore, we present evidence that the signal transduction of PPAR can be mediated by the presence of the estrogen receptor. We have verified the fidelity of our transcriptional reporter system using antisense expression vectors in transient transfection analysis and begun to examine the regulation of expression of this gene in breast cancer cells. These studies may lead to better understanding of the risk of specific dietary components as fatty acids can indeed function as hormones and further investigation could lead to changes in dietary guidelines. The implications of these studies could have a profound impact on both prevention and management of this devastating disease.				
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INTRODUCTION

The incidence of breast cancer has risen to become one of the leading causes of cancer related deaths among American women (1). While intensively investigated, the cause of this rise remains unexplained. Chinese women, however, have a significantly lower risk of breast cancer and this difference cannot be accounted for by genetic factors (2). Although epidemiological analysis suggests a correlation between high fat diets and breast cancer (3-9), much controversy remains. Animal studies, by contrast, have provided convincing evidence of a correlation between dietary fats, types of fats ingested and mammary tumors (10-13). In our efforts to determine if a molecular mechanism exists to link dietary fats to breast cancer incidence, we cloned the peroxisome proliferator-activated receptor gamma (PPAR γ) from several breast cancer cell lines (14). We have further demonstrated that individual fatty acids are able to positively or negatively mediate transactivation of PPAR in breast cancer cell lines (15). PPAR γ is a nuclear receptor with wide-ranging roles in tumor and normal development (16-18), atherosclerosis (19-21), angiogenesis (22) and adipocyte differentiation (23,24). Despite recent interest, its role in the ductile epithelia of the breast is not known. In breast cancer cells, PPAR transactivation has been reported to increase and decrease proliferation (15,25), induce apoptosis (25), and reduce their metastatic potential (26). They have also been reported to protect mice from chemically-induced mammary tumors (27) and induce primary human tumor cells to a more differentiated, less malignant state (28). The goal of this work is to determine whether PPAR expression and transactivation by dietary fatty acids is important in the etiology of breast cancer. To this end, we are examining the isoforms expressed, the regulation of expression and the molecular mechanism of transcriptional activation of PPAR using cell culture systems. These data could lead to new approaches in the treatment and prevention of breast cancer through the development of novel therapeutic interventions and revised dietary guidelines for women at risk of breast cancer as well as breast cancer survivors.

Body

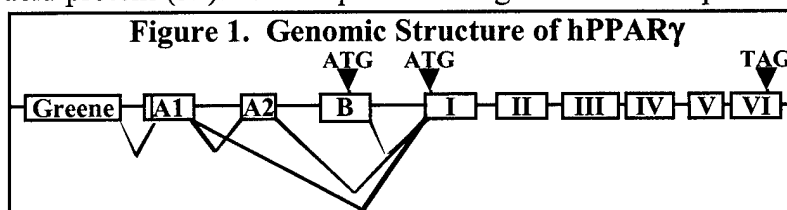
Research Accomplishments:

PPAR has three known members, α , β (also termed δ) and γ and within gamma, 3 more subtypes have been identified, $\gamma 1$, $\gamma 2$ and $\gamma 3$ (29-31). Using a mouse cDNA we originally determined that PPAR is expressed in both MCF-7 and T-47-D human breast cancer cells by Northern blot analysis. To confirm this observation and to identify the form(s) of PPAR expressed a cDNA library was constructed from MCF-7 cells and screened with both PPAR and RXR probes. These were designed against the DNA binding domain of the receptors, the most highly conserved portion of the gene, to identify all forms present. The isolated C1 clone was clearly PPAR γ , containing the ATG start site at base +173 (Accession # L40904) and the stop sign at base 1607 which encodes the full open reading frame of the 478 amino acid protein (32). Our sequence diverged from that reported by

Greene and co-workers immediately 5' of the start site of translation. An additional 74 bases were inserted between the untranslated exon A1 and the ATG-containing exon (exon I). This was later described as exon

A2 (30). As seen in figure 1, exon A2 is either a splice variant of $\gamma 1$ expression or can be the first untranslated exon and may be associated with unique promoter elements (31). PPAR $\gamma 1$ and 3 code for identical proteins while $\gamma 2$ contains an additional 28 N'-terminal amino acids. The functional significance of these differences remain unclear. None of the cDNA's we have isolated contains exon B which encodes these additional amino acids and RT-PCR using an exon B-specific oligonucleotide could detect no exon B usage in MCF-7 or MDA-MB-231 cells. For these reasons, we believe the only protein expressed in these cells is PPAR $\gamma 1$.

Since the genomic structure and thus the regulation of PPAR is complex, we sought to determine if other forms of PPAR are expressed in MDA-MB-231 cells which have not been previously reported. To this end we have performed 5' RACE (rapid amplification of cDNA ends). Thus far we have isolated 4 clones which are depicted in figure 2. In the top structure exon A1 is fused



to A2, then exon I, similar to previous reports (30). The second clone contains A2 fused to exon I but contains more than 180 bases of the 5' flanking sequence of A2 previously reported to be $\gamma 3$ promoter sequence (31). Since the RACE library was constructed from total RNA and the distance between exon's A2 and I are more than 20kb apart, it is very unlikely this represents contamination from genomic DNA. Thus it remains possible that transcriptional initiation in MDA-MB-231 cells lies further

5' of the transcriptional start site of A2 in adipocytes (31). The third clone contains an exon reported only in Rhesus monkeys (AF033342) fused to A2 and exon I (33). The human sequence we have cloned is 96% identical to that of the Macaque. The fourth clone we isolated is unique and has not been previously reported to Genbank. Currently the relative levels of expression driven from each promoter, the genomic structure of this gene and the functional significance of these findings are still unclear and the subject of further investigation.

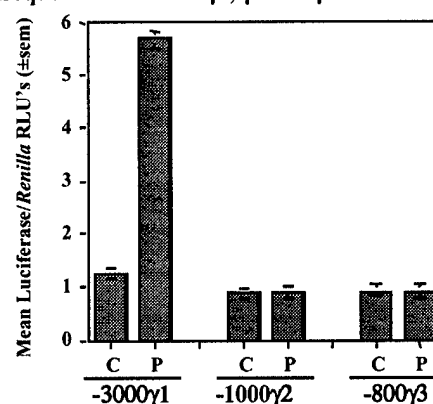
To assess the regulation of PPAR in human breast cancer cells, we have obtained three genomic reporter constructs which drive the tissue-specific expression of PPAR in adipocytes and hepatocytes

Figure 2. RACE Clones Isolated from MDA-MB-231 Cells

A1	A2	Exon I
5' flanking	A2	Exon I
AF033342	A2	Exon I
Unknown	A2	Exon I

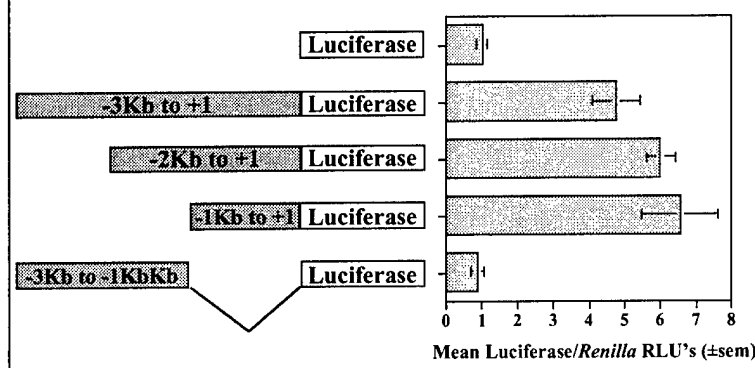
specific for $\gamma 1$, 2 and 3 (30,31). These contain 3000 bp upstream of A1, 1000 bp upstream of B and 800 bp upstream of A2, respectively, driving the expression of luciferase (a kind gift from Dr. Johan Auwerx, Institut de Genetique et Biologie Moleculaire et Cellulaire, Illkirch, France). These were transfected into both MCF-7 and MDA-MB-231 cells and relative luciferase activity compared to control plasmids containing the basic luciferase vector (Promega) lacking the 5' flanking sequences. The data shown in figure 3 is typical of what is seen in both cell lines. These data indicate that the 3000 bp flanking the 5' of exon A1 and not the elements upstream of B or A2, contains *cis*-regulatory elements capable of mediating basal expression of PPAR in both MCF-7 and MDA-MB-231.

Figure 3. Promoter Activity of the 5' Flanking Sequences of PPAR $\gamma 1$, $\gamma 2$ and $\gamma 3$ in MCF-7 Cells



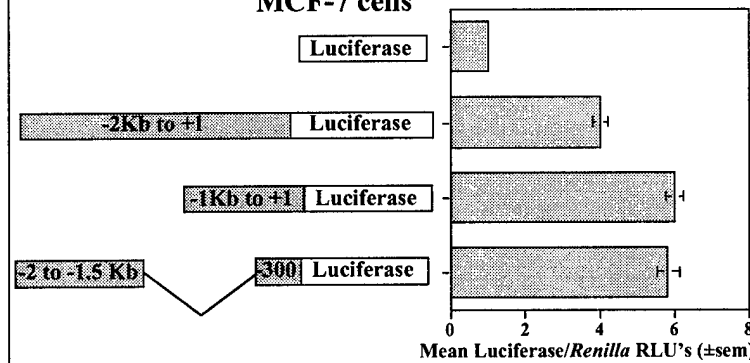
To begin to examine the sequences which confer basal expression of PPAR $\gamma 1$ in these cells, we have constructed deletion mutants of the 3 kb sequence flanking the 5' of the transcriptional start site and assessed their ability to mediate transcription of a reporter gene. Deletion mutations were created by restriction digestion and religation and all structures were confirmed by sequence analysis (figure 4). All were compared to the null vector containing the luciferase reporter but lacking the 5' flanking sequence. The entire 3 kb fragment confers basal transcriptional regulation of PPAR $\gamma 1$ and removal of the distal 1000 bp does not alter reporter activity. Removal of an additional 1000 bases does not have a

Figure 4. hPPAR $\gamma 1$ Deletional Analysis
MCF-7 cells



significant effect on basal expression, suggesting that the regulation of basal transcription in these cells is mediated by the proximal 1000bp. To further assess this hypothesis, an additional mutation was constructed lacking the proximal 1000 bp but containing the distal 2 kb removed in the prior construct (figure 5). This sequence appears to have no positive regulatory elements. Furthermore, the distal 500 bp between -2000 and -1500 fused to the proximal 300 bases contains the same reporter activity as that seen in the proximal 1000 bp. This suggests that no negative regulatory elements are present between -1500 and -2000 and that the positive basal regulation of PPAR $\gamma 1$ in these cells is contained in the proximal 300 bases of the promoter.

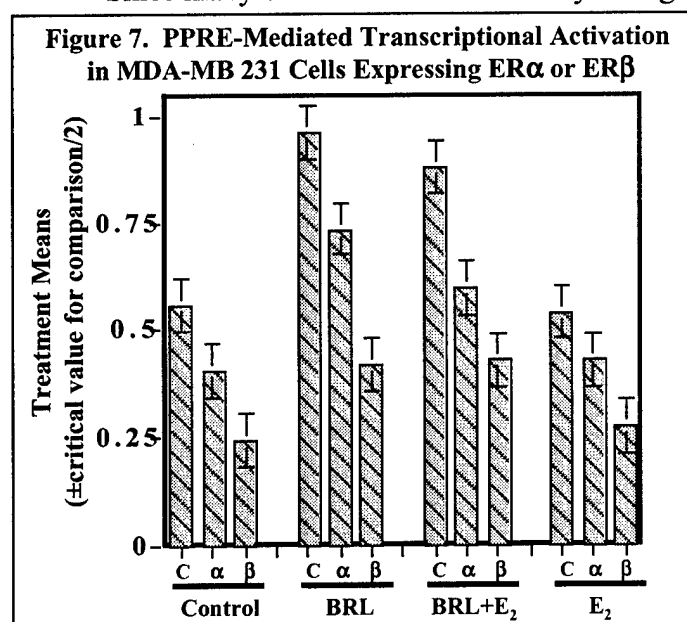
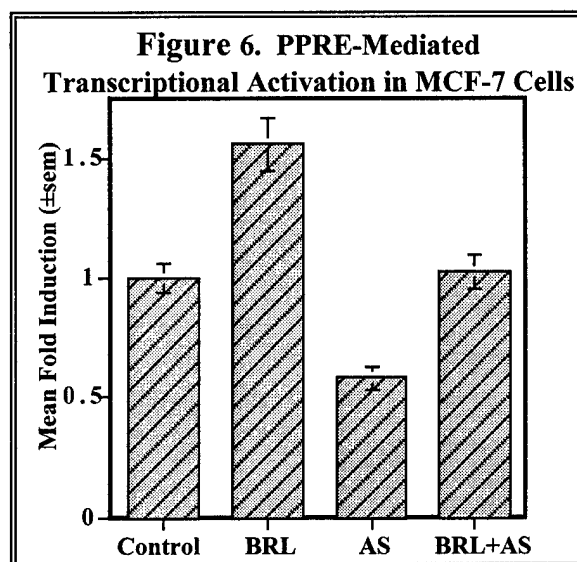
Figure 5. hPPAR $\gamma 1$ Deletional Analysis
MCF-7 cells



In contrast to the complex pattern of PPAR expression, a single cDNA species of RXR was isolated from both the MCF-7 and the MDA-MB-231 libraries. Despite using a degenerate probe that would have hybridized to all forms of RXR under the conditions used in Southern blot analysis, the

only cDNA identified was RXR α . A full-length cDNA was isolated and contained no differences from those reported previously (34).

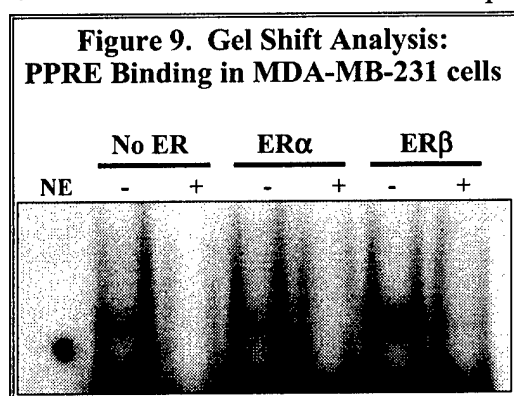
To examine the transactivation of PPAR γ 1 in breast cancer cells we have constructed transactional reporters containing peroxisome proliferator response elements (PPRE). These have been used in transient transfection assays to assess the transactivation of the PPAR-RXR complex. We have demonstrated that synthetic peroxisome proliferators increase the activity of transcriptional reporters containing either the peroxisome proliferator response element (PPRE) or the perfect direct repeat (DR-1) response element (14). Furthermore, we have gone on to demonstrate that individual fatty acids are capable of selectively functioning as agonists or antagonist of PPAR (15). This observation touches the central questions regarding whether PPAR mediates the effects of individual fatty acids present in diets and alters the incidence or growth rates of breast tumors. These data also indicate that PPAR is constitutively transcribed, translated and transactivated since treatment with ω -3 fatty acids reduced reporter activity to levels below control (see attached manuscript)s. Furthermore, transactivation of PPAR with fatty acids correlates with proliferation *in vitro*. It is not clear, however, whether this is a direct or indirect effect. To prove that the transcriptional reporter is indeed measuring the transactivation of PPAR we have constructed a PPAR γ antisense expression vector in order to block translation of the mRNA and thus reduce the basal levels of the protein. The antisense expression vector codes for an antisense mRNA spanning the sequence between the start of translation at base 173 and base 693 (accession # L40904). As seen in figure 6, the expression of the antisense mRNA selectively inhibited reporter activation while the sense vector had no effect (data not shown). Furthermore, the antisense expression not only inhibited activation of PPAR but it also inhibited basal transactivation again indicating that PPAR is constitutively transactivated in these cells.



Since many breast tumors are initially estrogen receptor (ER) positive and become ER negative and PPAR has been shown to mediate the transcriptional regulation of estrogen target genes, we have sought to determine whether either form of the ER (alpha or beta) participates in the transactivation of PPAR. MDA-MB-231 cells, which do not express ER, were transfected with the PPRE-mediated reporter with (α or β) or without (C) expression vectors for ER α or ER β . As seen in figure 7, the mere presence of the ER inhibited PPAR transactivation and inhibited transactivation by the synthetic PPAR ligand BRL 48,482 (BRL). Furthermore, this inhibition of PPAR transactivation is independent of ligand as the presence of 17 β estradiol (E₂) had no effects on ER inhibition of PPAR. Furthermore, the presence of the

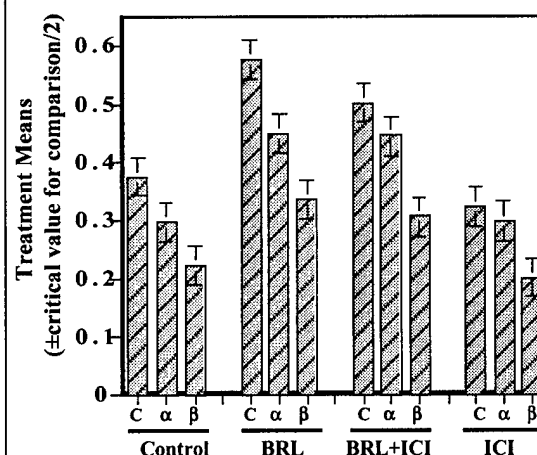
pure antiestrogen ICI 182,780 (ICI) was without effect regarding PPAR transactivation (fig 8). Coupled with the observations regarding PPAR mediated transcriptional regulation of ER target genes, these observations suggest that signal cross-talk exists bidirectionally between ER and PPAR and their respective response elements.

To examine the mechanism of the signal cross talk we have sought to determine the role of the ER in PPRE binding. Using gel shift analysis we have sought to determine whether the presence of either ER α or ER β alters the ability of PPAR to recognize and bind to a PPRE. MDS-MB-231 cells were transfected with either an ER α or ER β expression vector and nuclear proteins isolated from these untransfected cells. As seen in figure 9, the presence of either form to the ER does not prevent PPAR



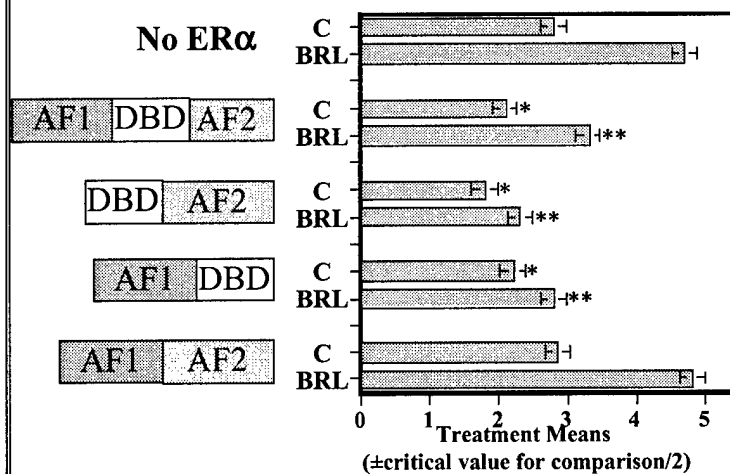
vectors which will code for proteins lacking the AF1, AF2 or the DNA binding domain. As shown in figure 10, MDA-MB-231 cells were transfected with the PPRE-mediated reporter alone or in conjunction with either the wild type ER α , or mutants lacking one of the three domain as shown. Although the AF1 and AF2 domains retained their ability to repress PPAR transactivation, deletion of the DBD rendered this construct ineffective. The mechanism of this effect and the role of the DBD in this process are still under investigation. Nevertheless, it is clear that the presence of a functional ER alters these cells ability to respond to PPAR-mediated changes in transcriptional regulation. The physiological consequences of these effects are also under further investigation. This observation could have important implications for breast cancer treatment as the use of antiestrogen such as Tamoxifen is widely employed. The functional significance of this observation and the molecular mechanisms of this effect are under investigation.

Figure 8. PPRE-Mediated Transcriptional Activation in MDA-MB 231 Cells Expressing ER α or ER β



from binding to the PPRE suggesting that this interaction is not due to competition for DNA binding. To further explore this possibility, we have gone on to examine the region of the ER necessary for this inhibition of PPAR transactivation. ER, like PPAR and the other nuclear receptors in this family, have domains which function in transactivation, AF1 and AF2, and DNA binding, DBD. Although gel shift analysis indicates that no direct exists between the ER and the PPRE, we have examined which of these domains might be involved in PPAR repression. To this end we have constructed expression vectors for the wild type ER and

Fig. 10. PPRE-Mediated Transcriptional Activation in MDA-MB 231 Cells Expressing ER α Mutants



In summary we have made significant progress toward all three specific aims outlined in the grant proposal. As stated in aim 1, we have determined the isoforms of both PPAR and RXR and have found new complexity in the regulation of PPAR which could have implications in the tissue-specific control of expression. Toward understanding the regulation of PPAR expression, aim 2, we have demonstrated that there is a significant level of basal PPAR expression in both MCF-7 and MDA-MB-231 cells. Furthermore, the sequence flanking the 5' start site of Exon A1 mediates basal PPAR expression in these cells. We are further defining this region to identify the *cis* regulatory elements responsible for this regulation. In defining the molecular mechanism of transactivation of PPAR, aim 3, we have identified a unique form of regulation which involves signal cross talk between PPAR and the estrogen receptor. The mechanism of this unexpected yet potentially important effect and the functional significance are currently under investigation. Clearly PPAR γ 1 is present in breast cancer cells and may provide a direct link between diet and the increased rate of breast cancers seen in this country. These studies may lead to better understanding of the risk of specific dietary components. The data make it clear that fatty acids can indeed function as hormones and this information could lead to important new discoveries impacting dietary guidelines and could be of significant therapeutic value. The implications of these studies could have a profound impact on both prevention and management of this devastating disease.

Methods

Cell Culture: MB-MDA-231, T-47D and MCF-7 were obtained from the ATCC (Rockville, MD) and maintained as described in the attached manuscripts.

Probe Construction: The mouse pSV-Sport/PPAR γ 2 plasmid was received as a kind gift from Dr. Bruce M. Spiegelman's lab at the Dana Farber Cancer Institute. The hRXR α expression vector (pCMV-RXR α) was a kind gift from Dr. Ron Evans (Howard Hughes Medical Institute, San Diego, CA). Primers were designed to each which amplified a 288 bp and a 321 bp fragment of the DNA binding domain of PPAR and RXR, respectively. The reaction utilized 40 ng of plasmid, 20 pmol of each primer, dNTP (10 mM each) at 0.2 mM, 1.5 mM MgCl₂, 1X final concentration polymerase buffer (Promega), and 2.5 units *Taq* Polymerase.

cDNA Library Construction: Using oligo dT mRNA a cDNA library was created using the Time Saver synthesis kit (Pharmacia) as per instructions. After the second strand synthesis, 5 units of T4 RNA Polymerase were added to the reaction for 1 h at 37°C. The cDNA was then ligated and packaged into the Lambda Zap Express vector (Stratagene) as per instructions. The library was titered at 1.3×10^8 plaque forming units/ml (PFU/ml) with $\leq 15\%$ non-recombinants.

Northern Blot Analysis: Please see attached manuscript, Kilgore *et al.*, MCE.

Transfection and proliferation: Please see attached manuscript, Thoennes *et al.*, In press.

Library Screening: A total of 2×10^5 PFU of phage were incubated at 200 RPM with the bacteria for 15 min at 37°C and plated. Plaques were lifted onto Hybond N+ membranes, denatured, UV crosslinked with 12,000 μ J (Stratagene) and air-dried. Membranes were probed following prehybridization and hybridization steps were performed twice in 2X SSC for 20 min each wash with a final wash in 1X SSC for 20 min. All washes were performed at 65°C. Membranes were then put under Hyperfilm (Amersham) and developed. Dilutions of the phage plugs were then used to plate out for the next round of screening. Third or fourth round of plating was performed to yield a single population of phage. Helper phage plasmid excision was performed as per instructions that resulted in a pBK-CMV plasmid with cDNA insert.

Sequencing: Plasmid DNA was purified via the alkaline lysis and T4 and T7 universal primers, found in pBK-CMV insert flanking regions, were used during the first round of sequencing. Sequencing reactions consisted of 400 ng plasmid DNA, 8 μ l of ABI Prism Ready Reaction mix (Perkin-Elmer/ABI), 5 pmol of primer, sterile distilled water to a total reaction volume of 20 μ l and an oil

overlay. Sequencing reactions were run on a ABI 373 Stretch sequencer (Applied Biosystems, Inc.). The primers were based on the published Human PPAR γ 2 sequence (Accession L40904) in Genbank and on sequences based on our clones.

RACE and RT-PCR: In both cases, single stranded cDNA was synthesized by MMLV reverse transcriptase from polyA⁺ RNA (Clontech). For the initial reaction an oligonucleotide was used at bases +221 to +243 at the 3' end of exon I. For the RACE library the tailing reaction was performed according to manufactures instructions. In the PCR phase of both reactions the 3' oligonucleotide was between bases +198 to +218 for increased specificity of the PCR reaction. All reactions were carried out following manufactures recommendations (Clontech). Total RNA was isolated from both MCF-7 and MDA-MB-231 cells. "Ready To Go You Prime First Strand Beads" (Pharmacia Biotech) were used for single-stranded cDNA synthesis, using 20 pmole of the reverse complement primer HPR4 (438-419 of L40904) or HPR4 -10 (428-409) (both are contained within exon II). For the 4 sets of PCR reactions, 20 pmole of each of four 5' primers were used: "5'Greene" (which starts at bp23 of L40904), A1 (starting at bp 95 of L40904), A2 (starting at bp 172) and B (starting at bp 95, (30)). PCR products were ligated into a TA 2.1 cloning vector (Invitrogen) and screened using the BPF1 probe (bp 199-216). Eleven positive clones from the A2 primed population, and eleven clones from the A1 primed population, were identified and sequenced.

Key Research Accomplishments:

- We have demonstrated the several human breast cancer cells express PPAR γ 1 and RXR α and are functionally responsive to peroxisome proliferators. While we were the first to demonstrate this, and the only group to clone these from breast cancer cells, several other groups have confirmed these findings by other means.
- We have shown that the transcriptional regulation of PPAR expression is under a complex and tissue-specific control. We have cloned one new untranslated first exon and identified another not previously reported in humans. Through subsequent analysis we have demonstrated that the gamma 1 promoter is the predominate driver of basal expression in breast cancer cells.
- We have shown that fatty acids are capable of functioning as ligands for PPAR and can mediate the transcription of target genes.
- We have demonstrated that individual fatty acids are selective modulators of PPAR transactivation which may help explain the difficulty in interpreting the role of diet and breast cancer from the epidemiological data.
- We have demonstrated that PPAR is transcribed, translated and constitutively transactivated in 2 separate lines of breast cancer cells.
- We have now shown that signal crosstalk exists bidirectionally between ER and PPAR.

Reportable Outcomes

Training and employment:

1. 4 MS degrees awarded and supported by this grant. All have remained in science.
2. 1 postdoctoral fellow recruited into the lab and into breast cancer research.
3. Based upon the work accomplished as a consequence of this grant I was recruited to the University of Kentucky School of Medicine in the Department of Molecular and Biomedical Pharmacology.

Manuscripts:

MW Kilgore, PL Tate, S Rai, E Sengoku and TM Price 1997 MCF-7 and T47D Human Breast Cancer Cells Contain a Functional Peroxisomal Response. *Molecular and Cellular Endocrinology*, 129:229-235.

SR Theonnes, PL Tate, TM Price and **MW Kilgore** 2000 Differential Transcriptional activation of peroxisome proliferator-activated receptor gamma by omega-3 and omega-6 fatty acids in MCF-7 cells.. *Mol Cell Endocrinol*. 2000 Feb 25;160(1-2):67-73

PL Tate, JM Nani TM Price and **MW Kilgore** 2000 Ginseng activates transcription of estrogen responsive genes through the alpha and beta forms of the estrogen receptor. (Submitted, *Journal of Endocrinology*).

X Wang, E Loghin, and **MW Kilgore** 2000 Signal cross-talk between the alpha and beta forms of the estrogen receptor and the peroxisome proliferator-activated receptor in human breast cancer cells. (in preparation).

Abstracts presented:

MW Kilgore, PL Tate, S Rai, E Sengoku and TM Price. 1997 MCF-7 and T47D Human Breast Cancer Cells Contain a Functional Response To Peroxisomal Proliferators. (Sponsor, DJ Fernandes) (88th annual meeting of the American Association of Cancer Research, San Diego, CA, April, 1997).

E. Sengoku and **MW Kilgore**. cDNA Cloning of Human Peroxisome Proliferator Activated Receptor from MCF7 Human Breast Cancer Cells. (79th Annual meeting of the Endocrine Society, Minneapolis, MN, June 1997).

PL Tate, TM Price and **MW Kilgore**. The Effects of Estrogen on DNA Binding and Transcription Activation of Peroxisome Proliferator Activated Receptor in Human Breast Cancer Cells. (79th Annual meeting of the Endocrine Society, Minneapolis, MN, June 1997).

S Rai, PL Tate and **MW Kilgore**. Dietary Fats Stimulate Peroxisome Proliferator Activated Receptor-Mediated Transcription in MCF7 Human Breast Cancer Cells. (79th Annual meeting of the Endocrine Society, Minneapolis, MN, June 1997).

E Sengoku and **MW Kilgore**. MCF-7 cells express a unique form of the peroxisome proliferator-activated. . Seventh annual South Carolina Research Conference, Wild Dunes Isle of Palms, SC, January, 1998.

S Rai and **MW Kilgore**. Selective activation of the peroxisome proliferator-activated receptor by dietary fatty acids in human breast cancer cells. Seventh annual South Carolina Research Conference, Wild Dunes Isle of Palms, SC, January, 1998.

PL Tate, JM Marchiori and **MW Kilgore**. *Panax ginseng* extract activates the estrogen receptor in breast cancer. (80th Annual meeting of the Endocrine Society, New Orleans, LA, June 1998).

Abstracts presented continued:

S Rai, PL Tate, TM Price, **MW Kilgore**. Role of dietary fats in PPAR-mediated carcinoma in MCF-7 human breast cancer cell lines. (80th Annual meeting of the Endocrine Society, New Orleans, LA, June 1998).

X Wang, PL Tate, TM Price and **MW Kilgore**. Signal cross talk between the estrogen receptor and the peroxisome proliferator-activated receptor in human breast cancer cells. Eighth annual South Carolina Research Conference, Wild Dunes Isle of Palms, SC, January, 1999.

PT Tate, JM Nani, TM Price and **MW Kilgore**. The response of breast cancer cells treated with ginseng extract. . (81st Annual meeting of the Endocrine Society, San Diego, CA, June 1999).

X Wang, PL Tate, TM Price and **MW Kilgore**. Signal cross talk between the estrogen receptor and the peroxisome proliferator-activated receptor in human breast cancer cells. (81st Annual meeting of the Endocrine Society, San Diego, CA, June 1999).

S Peirce and **MW Kilgore**. A Dominant Negative Mutant of PPAR γ Reduces PPRE-mediated Reporter Activity in MCF-7 Human Breast Cancer Cells. Ninth annual South Carolina Research Conference, Wild Dunes Isle of Palms, SC. January 2000.

ER Loghin and **MW Kilgore**. Regulation of Peroxisome Proliferator-Activated Receptor GAMMA (PPAR γ) Gene Expression in MCF-7 and MDA-MB-231 Human Breast Cancer Cells. Ninth annual South Carolina Research Conference, Wild Dunes Isle of Palms, SC. January 2000.

X Wang, PL Tate, SR Thoennes, E Loghin, TM Price and **MW Kilgore**. Signal Cross Talk between Estrogen Receptor Alpha and Beta and the Peroxisome Proliferator-Activated Receptor gamma1 in MDA-MB-231 Breast Cancer Cells. Era of Hope, Department of Defense Breast Cancer Research Program Meeting. Atlanta, GA, June 2000.

MW Kilgore, X Wang, SR, Thoennes, E Loghin and TM Price. Estrogen Receptor Alpha and Beta Mediate Transcriptional Activation of the Peroxisome Proliferator-Activated Receptor Gamma1 in MDA-MB-231 Breast Cancer Cells. (82nd Annual meeting of the Endocrine Society, Toronto, Canada, June 2000).

Invited presentations:

The molecular role of peroxisome proliferator-activated receptor on breast cancer in humans. University of Texas Southwestern Medical Center, Dallas, TX. April 1997.

Effects of Peroxisome Proliferators in the Human Breast: A Molecular Model for the Role of Diet in Breast Cancer. Carolinas Medical Center. Charlotte, NC, March 1998.

Peroxisome proliferator-activated receptor putative link between the environment and cancer. Medical University of South Carolina, August, 1998

Invited presentation Continued:

Effects of peroxisome proliferators on human breast cancer cells: A molecular model to examine the link between diet and breast cancer. University of Texas Health Center at Tyler. January 1999.

Effects of peroxisome proliferators on human breast cancer cells: A molecular model to examine the link between diet and breast cancer. Clemson University Institute for Environmental Toxicology. March 1999.

Molecular mechanisms of nuclear receptor transactivation in human breast cancers cells by dietary fatty acids and phytoestrogens. University of Louisville School of Medicine. April, 1999.

Molecular mechanisms of peroxisome proliferator-activate receptor transactivation in MCF-7 and MDA-MB-231 human breast cancer cells. Texas Tech University HSC. August, 1999.

Molecular mechanisms of nuclear receptor in human breast cancer cells by dietary acids and phytoestrogens. MD Anderson Cancer Center, Research Park, Department of Molecular Carcinogenesis. November, 1999

Description of Training:

To date four students have received their masters' degrees (MS) as a result of this award. Ms Sudha Thoennes is currently a technician at the University of Florida, Gainesville Medical School and is investigating viral mechanisms of oncogenesis. Mr. Eiichi Sengoku is currently Product Manager for North American Operations at Nalgen in Rochester New York. Major Mark Corbett is serving in the US Army in the Chemical Corp following completion of his degree. Ms. Evelina Loghin is a technician on a DARPA funded project at Clemson University's Department of Bioengineering. Ms. Rupalika Singh, an undergraduate student, has worked in the lab for the last 2 semesters has been accepted to medical school in part based upon her research experience gained in the my lab as a consequence of this grant support. Dr. Xin Wang has been in the lab as a postdoctoral fellow since October 1998 and has already presented two abstracts at international conferences. She continues to progress well and is currently writing a manuscript which will be submitted this spring. Also as a consequence of this grant and the data generated from its funding I was recruited to the University of Kentucky School of Medicine in the department of Molecular and Biomedical Pharmacology. This is a much better research facility with core facilities and collaborative faculty. I have also been accepted to the faculty of the Markey Cancer Center. I was also pleased to have Dr. Wang join me in this move and she continues to progress well.

Conclusions

These data indicate that human breast cancer cells express PPAR γ and respond to PPAR ligands by upregulating the expression of target genes. The implication of these findings is particularly important in light of the wide spread use of thiazolidinediones (TZDs) in the treatment of type-2 diabetes, which are themselves ligands for PPAR γ . Furthermore, we have demonstrated that dietary fatty acids shown to bind PPAR γ (35) can regulate transactivation in these cells. This suggests that fatty acids can function as hormones and mediate transcription of target genes in the breast. The fact that we observe selective activation by different classes of fatty acids may indicate that they have

differential functions in the breast. Whether these or other ligands play a role in breast cancer development or prevention must now be addressed.

Our demonstration that ER and PPAR γ signal transduction pathways interact could have profound implications in the treatment and prevention of breast cancer. Clearly, the ER status of a breast tumor is used as the principle indicator for treatment with Tamoxifen and other antiestrogen. What is not clear is whether all the therapeutic benefits from these drugs are direct effects through the ER or whether they are mediated through other receptor systems. The fact that ER downregulates the ability of PPAR γ to mediate gene transcription could have important physiological consequences and these must be examined. Furthermore, the role PPAR γ plays in normal development of the mammary gland and in the development or progression of breast cancer must too be examined. These studies could provide an important new target in our battle against breast cancer.

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Differential transcriptional activation of peroxisome proliferator-activated receptor gamma by omega-3 and omega-6 fatty acids in MCF-7 cells

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Abstract

While the role of dietary fats in breast cancer remains controversial, the recent cloning of peroxisome proliferator-activated receptor gamma (PPAR γ), a nuclear hormone receptor, from human breast cancer cells lines provides a potential molecular link. Several fatty acids from four classes of dietary fats were tested for their ability to mediate the transcriptional activity of PPAR γ in MCF-7 and MDA-MB-231 cells using growth media with minimal serum. Whereas omega-3 fatty acids inhibit transactivation of PPAR γ to levels below control, omega-6, monounsaturated and saturated fatty acids stimulate the activity of the transcriptional reporter. These studies indicate that individual fatty acids differentially regulate the transcriptional activity of PPAR γ by selectively acting as agonists or antagonists. Furthermore, the transcriptional activation of PPAR γ correlates with cell proliferation in MCF-7 cells. Understanding the effects of individual fats on breast cancer cells and PPAR γ transactivation could provide important new insights into the epidemiology of breast cancer and the role of dietary fat. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: PPAR γ ; Transcription; Breast cancer; Dietary fats; Omega-3 fatty acids; Omega-6 fatty acids

1. Introduction

Breast cancer is the leading cause of the cancer deaths among American women (Menck et al., 1997). In 1997, 180 200 women were diagnosed with breast cancer and approximately 43 900 women died of this disease in the US (Parker et al., 1997). In contrast one in 40 Chinese women will develop breast cancer and this difference cannot be accounted for by genetic factors (Ziegler et al., 1993). Although epidemiological analysis suggests a correlation between high-fat diets and breast cancer in humans (Kelsey and Gammon, 1990; Wynder et al., 1991; Hunter et al., 1996; Green-

wald et al., 1997; Rose, 1997; Rohan et al., 1998; Santiago et al., 1998; Simonsen et al., 1998; Snyderwine, 1998), controversy remains. Animal studies, however, have provided convincing evidence of this link and clearly demonstrate a correlation between dietary fats, types of fats ingested and mammary tumors (Karmali et al., 1984; Cohen et al., 1986; Freedman et al., 1990; Cohen et al., 1993; Parkinson et al., 1994; Rose and Hatala, 1994; Rose and Connolly, 1997).

It has been suggested that ω -6 fatty acids, which are high in Western diets, might be associated with higher risk of breast cancer incidences (Rose and Hatala, 1994; Rose, 1997). By contrast, populations whose fat intake is primarily ω -3 fatty acids have a lower incidence of breast cancer (Parkinson et al., 1994) and ω -3 fatty acids inhibit growth and metastatic potential of human cells in animal models (Karmali et al., 1984; Jurkowski

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et al., 1985; Cohen et al., 1993). The results from animal studies suggest that both the amount (Carroll and Braden, 1984; Aksoy et al., 1987; Katz and Boylan, 1989) and type (Braden and Carroll, 1986; Hubbard and Erickson, 1987; Kort et al., 1987; Abou-el-Ela et al., 1989; Cave, 1991; Rose and Connolly, 1993; Rose et al., 1993; Rose and Hatala, 1994; Hubbard et al., 1998) of fats consumed play a role in the susceptibility, growth and metastatic potential of both chemically induced and surgically implanted tumors.

The studies presented in this report were performed to determine whether differential effects of fatty acids on MCF-7 and MDA-MB-231 cells might be mediated by PPAR γ . Peroxisome proliferator-activated receptors are members of the nuclear hormone receptor family that play an important regulatory role in adipogenesis and lipid metabolism (Schoonjans et al., 1996; Spiegelman and Flier, 1996). These transcription factors control the expression of genes encoding enzymes in lipid metabolic pathways (Schoonjans et al., 1996; Spiegelman and Flier, 1996). Three genes encoding PPAR have been identified in mammals and are termed PPAR α , β/δ and γ (Schoonjans et al., 1996; Spiegelman and Flier, 1996). While the physiological ligand remains unresolved, both synthetic and natural ligands have been reported (Tontonoz et al., 1994; Forman et al., 1995; Kliewer et al., 1995; Schoonjans et al., 1996; Spiegelman and Flier, 1996; Lehmann et al., 1997). Recently we reported that several human breast cancer cell lines express PPAR γ and contain a functional response to synthetic peroxisome proliferators (Kilgore et al., 1997). This finding has been confirmed by others in humans (Elstner et al., 1998; Mueller et al., 1998) as well as mice (Gimble et al., 1998). In this report we demonstrate that individual fatty acids selectively function as agonists or antagonists of PPAR γ in MCF-7 and MDA-MB-231 cells and the activation of PPAR γ correlates with an increase in cell proliferation in MCF-7 cells. This model enables us to begin to examine the molecular mechanism whereby individual components from a complex diet might alter growth and development of breast cancer.

2. Materials and methods

2.1. Chemicals

All fatty acids, Calcium chloride, HeBS reagent chemicals (NaCl, KCl, Na₂HPO₄·2H₂O), Me₂SO, indomethacin and Bovine Serum Albumin (BSA) were purchased from Sigma (St. Louis, MO). Concentrated stocks of free fatty acids including the monounsaturated fatty acids, (MUFAs) and the polyunsaturated fatty acids (PUFAs) were prepared either in EtOH (Apper Alcohol, Kentucky) or Me₂SO and final con-

centrations were made by dilutions with the culture medium. Ly 171,883 (Eli Lilly, Indianapolis, IN) was dissolved in EtOH and the final concentrations of EtOH and Me₂SO in all conditions was 0.1%.

2.2. Cell culture

MCF-7 and MDA-MB-231 cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in the Dulbecco's modified Eagle's medium (DMEM) or improved MEM (IMEM), respectively (GibCo) containing 10% fetal bovine serum (FBS) at 37°C in 5% CO₂. Cells were gradually adapted to TCH[®] serum replacement medium (Celox Laboratories, Inc., St. Paul, MN) supplemented with 0.5% FBS (HyClone, Logan, Utah) for MCF-7 or without serum for MDA-MB-231 cells. All media lacked phenol red. Cells were routinely grown in T-75 flasks (Corning) and transferred to 6-well plates (Corning) 2 days prior to transfection. Cells were transfected at \approx 40–50% confluence and not allowed to go through more than 20 passages to minimize genetic drift inherent in culture system.

2.3. Reporter plasmid construction and transient transfection assays

The DR1-SV40-LUC, was constructed by annealing and ligating complementary 27 bp oligonucleotides containing single copy of underlined DR1 response element (5'-CGCGTGACCAGGTCAAAGGTCA CG-TTC) into the unique *Mlu*I-*Xho*I site of the pGL3-Promoter vector (Promega). The 3 \times PPRE-TK-luciferase construct was described previously (Kliewer et al., 1992). MCF-7 and MDA-MB-231 cells were transfected using the calcium phosphate method (Ausbel et al., 1994). Each well received 10 μ g 3 \times PPRE-luciferase plasmid DNA driven by a minimal TK promoter and 0.33 μ g β -galactosidase containing plasmid, constitutively driven by the CMV promoter. Following treatments, cells were lysed in 200 μ l lysis buffer and treated according to manufacturer's instructions (Analytical Luminescence Laboratory). Luciferase activity from 40 μ l of lysate was measured for 10 s (ALL) and 15 μ l lysate was used to measure β -galactosidase activity according to the Galacto-Light instructions (Tropix) for 5 s on a Monolight 2010 (ALL). The relative luminescent values for each well were derived by dividing the inducible luciferase values by the constitutive β -galactosidase values. Stock solutions of omega-3 PUFA, omega-6 PUFA and monounsaturated fatty acids were made in absolute ethanol, and the working solutions were made by dilutions in TCH medium. Stock solutions of saturated fatty acids were prepared in Me₂SO (DMSO) and the working solutions were made in TCH medium containing BSA at a final con-

centration of 0.2%. Each set of treatments was performed in triplicate in three or more independent experiments. Statistical analysis is a randomized block design. Treatment means were compared pairwise using Tukey's multiple comparison analysis and confidence limits were set at $P \leq 0.05$. Data are plotted as the treatment means and the error bars are plus and minus one half of the critical value for comparison, thus error bars that do not overlap represent statistically significant differences.

2.4. Cell proliferation assay

Proliferation was determined by measuring the incorporation of tritiated thymidine into cellular DNA. Thirty hours after treatment 1.0 μCi [^3H] thymidine was added to each well and cells incubated for an additional 6 h. Each well was washed four times in PBS and the adherent cells were lysed in 500 μl of 1 \times trypsin-EDTA and 500 μl of a lysing solution (2 N NaOH, 6.82 mM, *N*-lauroyl sarcosine and 10 μM EDTA). Lysates were individually transferred into 15 ml centrifuge tubes (Fisher) containing 100 μl of 0.5% Phenol red (Sigma) and 150 μl of formaldehyde, 37% w/w (Fisher). Samples were adjusted to a pH of 7.4 as determined by colorimetric analysis against a known standard using HCl and NaOH. Samples from each culture well were passed through a 0.45- μm filter (Millipore) using a 12-filter Millipore manifold. Filters were air-dried, placed in vials with 8 ml of Scintiverse scintillation fluid and counted on a Beckman LS6500. Each set of treatments were performed in replicates of five in three independent experiments. The experimental design is a randomized block design. Treatment means were compared pairwise using Tukey's multiple comparison anal-

ysis and confidence limits were set at $P \leq 0.05$. Data are plotted as the treatment means and the error bars are plus and minus one half of the critical value for comparison, thus error bars that do not overlap represent statistically significant differences.

3. Results

To determine whether PPAR γ activated the DR-1 and 3 \times PPRE constructs, both were tested in MCF-7 cells. The synthetic peroxisome proliferator LY 171 883 stimulated reporter activity in cells transfected with either the PPRE or the DR-1 equally (data not shown). Furthermore, the null vectors lacking the DR-1 or the three PPRES were unresponsive indicating the cloned response elements mediated these effects (data not shown).

Next we examined the ability of individual fatty acids to function as ligands and mediate the transcriptional regulation of PPAR γ . MCF-7 cells were transiently transfected with the 3 \times PPRE-TK-luciferase and treated individually with omega-3 PUFA, omega-6 PUFA, monounsaturated fatty acids or saturated fatty acids. In all cases, the fatty acid concentrations used in these studies were those shown to be maximally effective in this culture system. This was determined over a range of fatty acid concentrations. Of the omega-3 PUFAs, linolenic acid (LnA, C18:3 ω 3), eicosapentaenoic acid (EpA, C20:5 ω 3), docosahexaenoic acid (DhA, C22:6 ω 3) and docosapentaenoic acid (DpA, C22:5 ω 3) were tested. The results in Fig. 1 demonstrate that omega-3 PUFA inhibit the transcriptional activity of PPAR γ to levels below control. The strongest inhibition was observed by EPA (100 μM), which inhibited reporter activity to 66% ($P \leq 0.001$) relative to control. Linolenic acid resulted in 80% reporter activity at 10 nM ($P \leq 0.002$) relative to control. DhA also inhibited PPAR γ activity to 89% of control at 10 μM concentration ($P \leq 0.02$). By contrast, DpA did not inhibit the transcriptional activity of PPAR γ . Similar results were obtained in another set of experiments in which MCF-7 cells were transfected with DR1-SV40-LUC reporter construct and treated with omega-3 PUFAs (data not shown).

Three omega-6 PUFAs, linoleic acid (LaA, C18:2 ω 6), arachidonic acid (ArA, C20:4 ω 6) and γ -linolenic acid, (γ -LnA, C18:3 ω 6) were tested (Fig. 2). Gamma-Linolenic acid (200 μM) stimulated reporter activity 1.63-fold induction ($P \leq 0.005$) while linoleic acid (250 μM) resulted in a 1.57-fold induction ($P \leq 0.005$) and arachidonic acid (250 μM) produced a 1.52-fold induction ($P \leq 0.005$). Similar results were seen in MDA-MB-231 cells where linoleic acid (250 μM) significantly induced reporter activity of the 3 \times PPRE-luciferase reporter 4.2-fold over control ($P \leq 0.001$, data not shown).

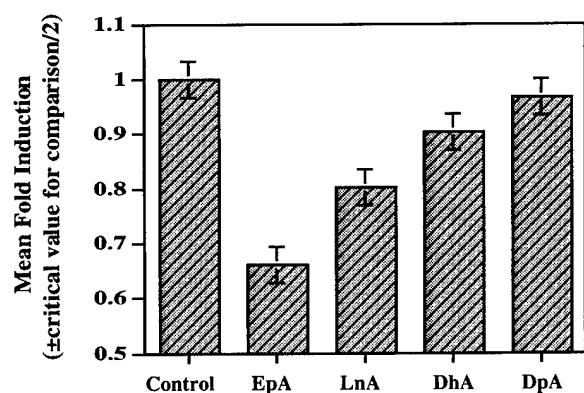


Fig. 1. Effects of omega-3 fatty acids on the transcriptional activity of PPAR γ in MCF-7 cells. Cells were transiently transfected with reporter plasmid and treated with vehicle alone (control), eicosapentaenoic acid (EPA), linolenic acid (LnA), eicosahexaenoic acid (DhA) or docosapentaenoic acid (DpA) for 18–24 h. Following ANOVA, data are plotted as the treatment means (\pm one half of the critical value for comparison). Error bars that do not overlap represent statistically significant differences ($P \leq 0.05$).

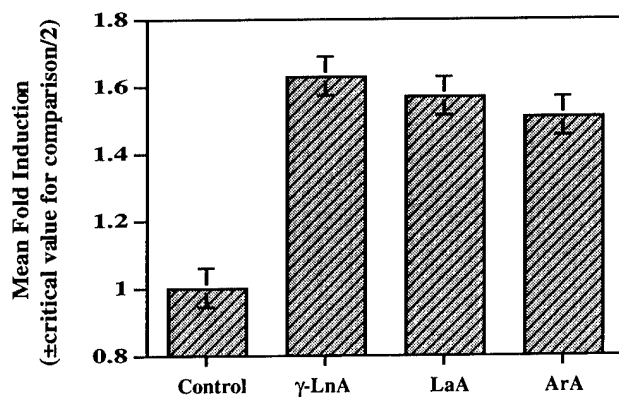


Fig. 2. Effects of omega-6 fatty acids on the transcriptional activity of PPAR γ in MCF-7 cells. Cells were transfected with the reporter plasmid and treated with vehicle alone (control), or linoleic acid (LaA), arachidonic acid (ArA) or γ -linolenic acid (γ -LnA), for 18–24 h. Following ANOVA, data are plotted as the treatment means (\pm one half of the critical value for comparison). Error bars that do not overlap represent statistically significant differences ($P \leq 0.05$).

Fig. 3 demonstrates the effect of two monounsaturated fatty acids (MUFAs), oleic acid (C18:1 ω 9) and petroselinic acid (C18:1 ω 12), on PPRE-mediated reporter activity in MCF-7 cells. Oleic acid (350 μ M) increased reporter activity 1.24-fold ($P \leq 0.05$) whereas petroselinic acid (150 μ M) stimulated reporter activity 1.85-fold ($P \leq 0.001$).

Five saturated fatty acids with increasing chain lengths, caprylic (C10:0), palmitic (C16:0), stearic (C18:0), arachidonic (C20:0) and lignoceric acid (C24:0) were tested (Fig. 4). Both caprylic (10 μ M) and palmitic acid (10 μ M) weakly stimulated reporter activity 1.15-fold ($P \leq 0.02$) and 1.11-fold, respectively. Lignoceric acid treatment (50 μ M) stimulated reporter activity 1.25 over control ($P \leq 0.05$). A 1.2-fold induction ($P \leq 0.05$) was seen with arachidonic acid treatment (100 μ M) and stearic acid (250 μ M) increased reporter activity 1.4-fold ($P \leq 0.002$).

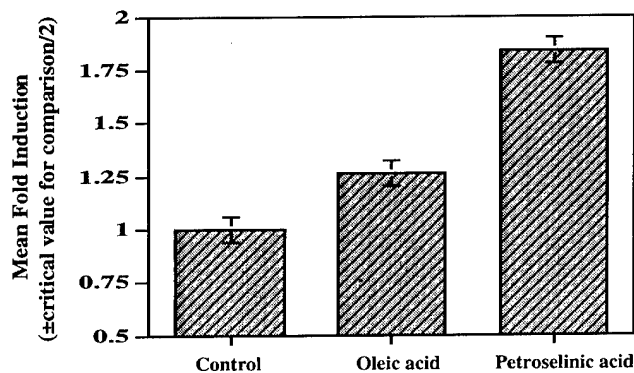


Fig. 3. Effects of monounsaturated fatty acids on the transcriptional activity of PPAR γ in MCF-7 cells. Following transfection, cells were treated for 18–24 h with vehicle alone (control), oleic or petroselinic. Following ANOVA, data are plotted as the treatment means (\pm one half of the critical value for comparison). Error bars that do not overlap represent statistically significant differences ($P \leq 0.05$).

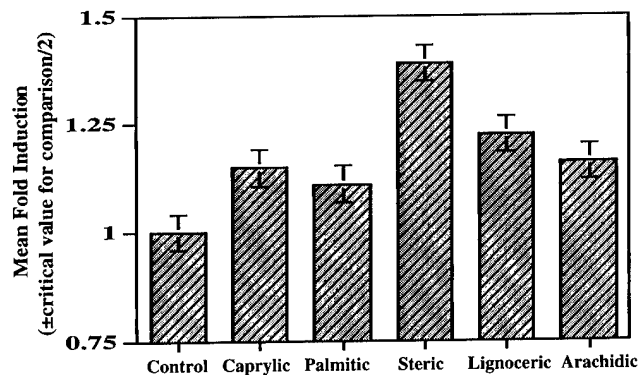


Fig. 4. Effects of saturated fatty acids on the transcriptional activity of PPAR γ MCF-7 cells. MCF-7 cells were transfected with the reporter plasmid and treated with vehicle alone (control), caprylic, palmitic, stearic, arachidic or lignoceric acid, as described, for 18–24 h. Following ANOVA, data are plotted as the treatment means (\pm one half of the critical value for comparison). Error bars that do not overlap represent statistically significant differences ($P \leq 0.05$).

Using the same concentrations reported in the transcriptional assays, the effects of linoleic and linolenic acids were examined for their ability to induce proliferation (Fig. 5). Estrogen, in the form of 17 β -estradiol, a known mitogen in MCF-7 cells, significantly increased cell proliferation as did linoleic acids ($P \leq 0.001$ and $P \leq 0.005$, respectively). By contrast, linolenic acid significantly inhibited cell proliferation to levels below control ($P \leq 0.01$).

4. Discussion

In an attempt to further clarify the role of individual dietary components on the physiology of human breast cancer cells, we have tested a variety of fatty acids for

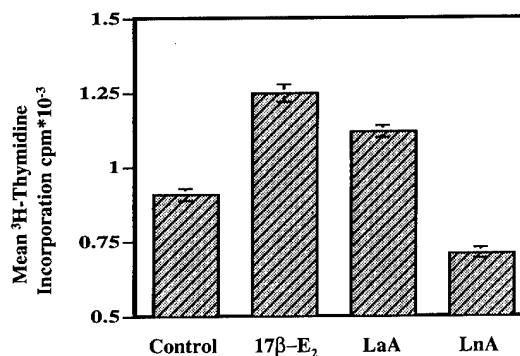


Fig. 5. Effects of 17 β -estradiol (E₂), linoleic and linolenic acid on MCF-7 cell proliferation. Cells were plated at equal densities and treated for 30 h with physiological concentrations of estradiol or of fatty acids at the same concentrations used in transfection experiments. Each well received [³H]thymidine plus treatments for an additional 6 h. Following ANOVA, data are plotted as the treatment means (\pm one half of the critical value for comparison). Error bars that do not overlap represent statistically significant differences ($P \leq 0.05$).

their ability to modulate the transcriptional activity of PPAR γ . Three important observations have been made as a consequence of these studies. First, individual fatty acids appear to selectively function as agonists or antagonists of PPAR γ in MCF-7 cells. Although variability exists between individual fatty acids within a class, clear differences exist between the classes of fatty acids themselves. These data suggest that transactivation of PPAR γ may be one mechanism whereby individual fatty acids can mediate cell-specific function by modulating gene expression. The fact that reporter activity was also stimulated in MDA-MB-231 cells with linoleic acid indicates this is not a response specific to MCF-7 cells and may be more generally applicable to other breast cancer cells. Secondly, while we have previously demonstrated that MCF-7 cells express high levels of mRNA, these data suggest that PPAR γ is constitutively transactivated in MCF-7 cells. This is supported by the observation that ω -3 fatty acids such as linolenic acid inhibits the activity of the transcriptional reporter to levels below that of control. This suggests that linolenic acid can compete with some endogenous ligand thus inhibiting basal activity. Thirdly, linoleic acid, an agonist of PPAR γ function, increases the rate of cell proliferation while linolenic acid treatment, a PPAR γ antagonist, reduces proliferation. This is in contrast to troglitazone, a synthetic agonist of PPAR γ , which has been reported to inhibit proliferation of 21MT cells (Mueller et al., 1998) and induce apoptosis when used in combination with all-*trans*-retinoic acid in MCF-7 cells (Elstner et al., 1998). Evans and co-workers, however, have demonstrated that activators of PPAR γ increase the incidence of colorectal tumors and polyp formation indicating that transactivation of PPAR γ may indeed result in tumor formation (Saez et al., 1998). In support of our proliferation studies animals fed linolenic acid have fewer tumors and a reduced metastatic potential when compared to animals maintained on diets rich in linoleic acid (Cave, 1991; Rose et al., 1994). The differences between the *in vitro* and *in vivo* data could be a function of cell-specific responses or due to differences in the ligand-receptor interactions. Although these data do not prove a cause and effect relationship between transactivation of PPAR γ and the control of cell cycle, the mechanism of this effect in MCF-7 cells clearly warrants further investigation.

In NIH 3T3 cells, a mouse adipose stromal cell line, transfection with PPAR γ is both necessary and sufficient to set in motion the differentiation into an adipocyte. The presence of PPAR γ in human breast cancer cells has led to the speculation that its transactivation could be used therapeutically to induce the re-differentiation of malignant cells into a benign state (Mueller et al., 1998). Here we report that MCF-7 cells express PPAR γ and the receptor exists in some continuous state of transcriptional activation, yet these cells do

not differentiate into adipocytes. This suggests that the transactivation of PPAR γ in MCF-7 cells sets in motion a program unique from that seen in adipose stromal cells. Clearly, it will be important to examine the genes regulated by PPAR γ in human breast cancer cells to understand the functional significance of these observations. Additionally, further studies will be necessary to determine the molecular mechanisms of tissue-specific responses.

A question critical to determining the role PPAR γ plays in human breast cancer is to assess its expression in normal ductile epithelia as well as primary and metastatic tumors. Gimble et al. (1998) reported that PPAR γ is expressed in ductile epithelia of virgin mice and rats. However, PPAR γ is not expressed during lactation nor in mammary tumors induced by 7,12-dimethylbenz(a)anthracene (Gimble et al., 1998). Furthermore, NmuMG, a normal mouse epithelial line, expresses PPAR γ but its expression is not inducible by peroxisome proliferators. In contrast to the rodent model, PPAR γ is expressed in several human cancer cell lines including T-47-D, MDA-MB-231, SK-BR3, ZR-75-1 and BT-20 cells (Kilgore et al., 1997; Mueller et al., 1998) and a functional response to peroxisome proliferators has been demonstrated in MCF-7 (Kilgore et al., 1997) and 21MT cells (Mueller et al., 1998). Finally, Spiegelman and coworkers have shown that PPAR γ is expressed in both primary breast tumors and in lung sections of patients with metastatic tumors (Mueller et al., 1998). The expression of PPAR γ in both benign and malignant cells leaves open the possibility that peroxisome proliferators could play a role in both normal and cancerous tissue.

The role of lipid in breast disease has been the subject of intense debate and, more recently, intensive investigation. The identification of a functional response to peroxisome proliferators in a wide variety of breast cancer cell lines and the ability of fatty acids to selectively mediate the transactional activity of PPAR γ lends further support for a direct role of dietary fatty acids in human breast cancer. Clearly, the functional significance of these findings will require further investigation.

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